Fundamental improvement of resolution with a 4Pi-confocal fluorescence microscope using two-photon excitation

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A new type of confocal fluorescence microscope with an unprecedented resolution is proposed. Calculations demonstrate that an axial resolution on the order of 100 nm can be achieved in a confocal microscope with enlarged aperture when two-photon excitation is applied. This axial resolution is up to four times higher than that of a confocal fluorescence microscope. The proposed microscope yields the highest point resolution ever achieved in far-field light microscopy.

1. Introduction

In confocal fluorescence microscopy, point-like illumination and detection are applied. The point-like illumination source and the point detector are focused on the object by a microscope objective. Thus, the point spread function (PSF) of a confocal fluorescence microscope is given by the product of the point spread functions for illumination and detection. The confocal point spread function leads to a discrimination of object points out of the focal area and a marginally higher resolution than its conventional counterpart [1-5]. In many practical systems, however, this slight enhancement is lost due to the finite size of the detector openings [6]. Resolution is quantified by extent of the confocal point spread function (PSF). In confocal microscopy resolution is equivalent to discrimination as only the light intensity originating from the focal area is measured.

For light microscopes using objective lenses, the axial resolution is one third or less of that in the lateral direction, depending on the numerical aperture [7]. This is especially unfortunate for the confocal microscope because axial resolution is one of the main advantages of this technique. Therefore the enhancement of the axial resolution of confocal microscopes has always been of considerable interest. Several methods to enhance the axial resolution have been suggested [8-14].

In this paper, a fluorescence microscope with an axial resolution even surpassing the lateral resolution is proposed. Its basic theory is presented and the resolution is compared with that of a confocal and two-photon (confocal) fluorescence microscope for a practical implementation.

2. Point spread function for high apertures

In far-field microscopy imaging is accomplished by focusing. If aberrations are neglected, the focusing process is described by the propagation of a calotte of an ideal spherical wavefront toward its center (fig. 1). The size of the calotte is determined by the aperture angle. To achieve a high resolution, large aperture angles are needed [7]. To calculate the point spread function the theory therefore must consider the electromagnetic properties of the incident light wave. According to Richards and Wolf [15], the normalized electric field for a linearly polarized incident wavefront $E$ is given by

$$E(u, v, \phi) = (e_x, e_y, e_z) = -i(l_0 + l_2 \cos 2\phi, l_2 \sin 2\phi, -2i l_1 \cos \phi),$$

where $\phi$ denotes the angle between the plane of vibration of the incident electric field and the plane of
The spread function of linearly polarized light in the focal region can be expressed by
\[ I_s(u, v, \phi) = |E|^2 = |I_0|^2 + |I_2|^2 + 4|I_1|^2 \cos^2\phi + 2\text{Re}\{I_0 I_2^*\} \cos 2\phi, \] (4)

Re denotes the real part [16]. For random polarization, the dependency on the azimuth \( \phi \) vanishes and the normalized point spread function is therefore
\[ I_r(u, v) = |I_0|^2 + 2|I_1|^2 + |I_2|^2. \] (5)

3. Theory of confocal, two-photon and 4Pi-confocal fluorescence microscopy

3.1. Confocal microscopy

The confocal fluorescence PSF is given by the product of the illumination and detection PSFs. If linearly polarized illumination is assumed, the normalized illumination PSF is equal to the intensity distribution of eq. (4). When random polarization of the fluorescent light is assumed the normalized detection PSF is given by eq. (5) but with a longer mean wavelength. Thus, the resulting confocal PSF is
\[ h_{\text{conf}}(u, v, \phi) = h_{\text{ill}}(u, v, \phi) h_{\text{det}}(u', v') = I_r(u, v) I_r(u', v') \approx h_{\text{ill}}^2(u, v). \] (6)

The approximation on the right is valid for equal illumination and fluorescent wavelengths and negligible polarization effects. The multiplication or squaring is the basic physical phenomenon in confocal imaging and is responsible for the confocal properties.

3.2. Two-photon fluorescence microscopy

In two-photon fluorescence microscopy, the excitation of the fluorophore is accomplished by two photons having only half of the energy (twice the wavelength) required to overcome the gap between the ground and the excited state. It was proposed by Sheppard and Kompfner [17] along with other nonlinear modes. Recently, Denk et al. [18] demonstrated the practical realization of two-photon laser
scanning fluorescence microscopy for biological and other applications. The major advantages are the restriction of photo-bleaching to the vicinity of the focus and the capability of producing confocal imaging properties (e.g., sectioning) without using a point detector. Since two photons are needed simultaneously for excitation, the PSF in two-photon microscopy is given by

$$h_{\text{two phot}}(u, v, \phi) = h_{\text{ill}}^2(u, v, \phi) = I_r(u, v, \phi).$$

(7)

When a point detector is added in a confocal arrangement, the PSF is given by

$$h_{\text{conf phot}}^2(u, v, \phi) = h_{\text{ill}}^2(u, v, \phi) \ h_{\text{det}}(u', v')$$

$$= I_r(u, v, \phi) \ I_r(u', v').$$

(8)

Due to this additional factor, a further increase in resolution with respect to the non-confocal arrangement is expected [18].

The resolution of two-photon confocal and non-confocal microscopy was evaluated by Sheppard and Gu [19] using scalar approaches which are applicable for lower numerical apertures. Furthermore, they assumed equal excitation and fluorescent wavelengths. They found that two-photon fluorescence microscopes have a lower point resolution than equivalent confocal fluorescence microscopes.

3.3. 4Pi-confocal fluorescence microscopy

In the two above-mentioned imaging techniques, the axial resolution is lower than the lateral resolution. This is due to the elongation of the PSFs in the axial direction (see figs. 2a). An intuitive explanation for the elongation along the optical axis is that the converging spherical wavefront is only a section of a complete spherical wavefront (fig. 1). If a complete spherical wavefront with an uniform amplitude were used, the PSF would have a spherical shape and the elongation would vanish.

Following this reasoning, the aperture of a confocal fluorescence microscope can be enhanced with a second microscope objective placed opposite to the first one (fig. 1). When both illuminate the common focal point with coherent wavefronts and constructive interference takes place, the illumination PSF approaches a more spherical shape and the axial resolution is enhanced. A similar consideration applies also to the confocal detection. Provided that the detected light interferes in the common point detector the detection aperture is enhanced. A technique relying on this principle has been first described by Hell in ref. [9]. Its feasibility has been demonstrated [13,14] for interferent illumination wavefronts. The technique was named 4Pi-confocal microscopy. When interferent illumination wavefronts are used with normal detection, it is called type A 4Pi-confocal microscopy. When additional interferent detection wavefronts are employed it is referred to as type C 4Pi-confocal microscopy. A somewhat related coherent mixing of images in a double pass confocal microscope was also speculated in ref. [12] and shown for reflection microscopy in ref. [11].

In a type A 4Pi-confocal fluorescence microscope the illumination PSF is given by

$$h_{\text{ill}}(u, v, \phi) = |(E_1(u, v, \phi) + E_2(-u, v, \phi))|^2$$

$$= \text{const} (\Re{I_0})^2 + \Re{I_2}^2$$

$$+ 2\Re{I_0} \Re{I_2} \cos 2\phi + 4\Re{I_1}^2 \cos^2 \phi,$$

(9)

where $E_1$ and $E_2$ denote the electric fields from the upper and lower objective lenses, respectively. The type A 4Pi-confocal PSF is given by

$$h_{\text{conf A}}(u, v, \phi) = h_{\text{ill}}(u, v, \phi) \ h_{\text{det}}(u', v')$$

$$= h_{\text{ill}}(u, v, \phi) \ I_r(u', v').$$

(10)
4. Resolution performance of the confocal, two-photon and 4Pi-confocal microscope

To compare the resolution performance of confocal fluorescence, two-photon fluorescence and type A 4Pi-confocal fluorescence microscopy the respective PSFs have been evaluated for the highest possible numerical aperture, namely NA=1.4 (oil immersion). In addition, the excitation and fluorescence wavelengths of Coumarin 400 have been chosen because this fluorophore is reported to possess two-photon excitation capability [18]. Coumarin 400 can be excited with a single photon at $\lambda_{\text{exc}}=350\,\text{nm}$ and, therefore, with $\lambda_{\text{exc}}=700\,\text{nm}$ in the two-photon mode. The mean fluorescence wavelength $\lambda_{\text{det}}$ is 440 nm [20].

The calculations were performed numerically using the formulas (6)–(10). Figure 2a shows the contour plot of the confocal fluorescence PSF $h_{\text{conf}}(u, v, \phi=\pi/2)$ normalized to unity. The bold horizontal axis represents the focal plane and the perpendicular to the direction of vibration ($\phi=\pi/2$) of the incident electric field for all figures. Since the PSFs are symmetrical with respect to the optical axis and the focal plane only one quarter of a full section along the optical axis is shown. The first minima in axial direction are located at $a=\pm 471\,\text{nm}$. Figure 3a displays the two-photon fluorescence PSF $h_{\text{two} \, \text{phot}}(u, v, \phi=\pi/2)$. Figure 3b exhibits the corresponding two-photon confocal fluorescence PSF $h_{\text{conf} \, \text{two} \, \text{phot}}(u, v, \phi=\pi/2)$ which is valid when a detector pinhole is used. Figures 2a, 3a and 3b confirm the prediction of the scalar calculations by Sheppard and Gu that the two-photon microscope has inherently a lower resolution owing to its doubled excitation wavelength $\lambda_{\text{det}}$ is 440 nm [20].

This is not the case in fig. 2b where the contour plot of the type A 4Pi-confocal fluorescence PSF $h_{\text{conf} \, \text{A}}(u, v, \phi=\pi/2)$ is shown. The 4Pi-effect can be distinguished clearly: the first minima are situated at $z=\pm 79\,\text{nm}$ instead of $z=\pm 471\,\text{nm}$ as in fig. 2a, promising an tremendous increase in axial resolution. However, the secondary maxima at $z=\pm 146\,\text{nm}$ reach the considerable relative height of 0.43. These secondary maxima reduce the benefit of the decrease in full-width-half maximum of the main peak by causing ghost images in a practical application.

In conclusion, figs. 2 and 3 reveal that two-photon fluorescence microscopy is not able to increase the resolution with respect to confocal microscopy as long as it is possible to excite using the shorter wavelength in the confocal mode. In contrast, 4Pi-confocal fluorescence microscopy has a strong potential to increase the axial resolution provided that the axial secondary maxima are reduced or at least taken into account mathematically.

5. Two-photon 4Pi-confocal microscopy

The following calculations demonstrate that the axial secondary maxima occurring in 4Pi-fluorescence microscopy can be reduced drastically when two-photon excitation is applied. According to eqs. (7) and (9), the two-photon 4Pi illumination PSF is given by

$$h_{\text{ill} \, \text{two} \, \text{phot}}(u, v, \phi) = h_{\text{ill} \, \text{conf}}^2(u, v, \phi),$$

and is shown in fig. 4a, calculated for a 700 nm excitation wavelength and two objective lenses with NA=1.4. The longer wavelength doubles the width of the PSF but squaring the PSF reduces the relative
height of the secondary maxima to 0.34. For two-photon 4Pi-confocal fluorescence microscopy, however, the resolution is determined by the product of the 4Pi-illumination PSF of fig. 4a and the corresponding detection PSF shown in fig. 4b:

\[ h_{\text{conf, A}}^{\text{two phot}}(u, v, \phi) = h_{\text{illum}}^{\text{two phot}}(u, v, \phi) h_{\text{det}}(u', v') \]. (12)

In contrast to normal fluorescence microscopy, the fluorescence wavelength (440 nm) is 1.6 times shorter than the illumination wavelength (700 nm). Therefore, the detection PSF \( h_{\text{det}}(u', v') \) is confined to a much smaller region than the illumination PSF. This results in a further marked reduction of the secondary maxima in axial direction.

The PSF for two-photon 4Pi-confocal microscopy \( h_{\text{conf, A}}^{\text{two phot}}(u, v, \phi) \) is shown in fig. 5. The full-width-half-maximum of the central peak in the axial direction is 108 nm. The axial secondary maxima have a relative height of only 0.096, which is about one fourth of the former height of 0.43 in a single-photon excitation 4Pi-confocal microscope (type A). Thus, the strong increase in axial resolution can be exploited.

In many situations stacked object layers must be separated. To quantify the axial resolution of planes perpendicular to the optical axis, the z-response of an infinitely thin fluorescent layer was calculated:

\[ I_{\text{layer}}(z) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} h(x, y, z) \, dx \, dy \]. (13)

Figure 6 displays the z-response for the proposed two-photon type A 4Pi-confocal fluorescence microscope (bold curve) along with the z-responses of the other
imaging modes. The full-width-half-maximum of the main peak is 110 nm and the height of the secondary peaks is 0.139. The confocal fluorescence microscope has a full-width-half-maximum of 310 nm, which is about three times larger, despite the fact that an excitation wavelength at half the size is used. A comparison of the confocal fluorescence and the two-photon confocal fluorescence shows that the axial discrimination capability of the latter is about 12 percent lower.

6. Conclusion

Figures 5 and 6 reveal that the two-photon type A 4Pi-confocal fluorescence microscope exhibits an axial resolution which is about three times better than that of a comparable confocal fluorescence microscope. For Coumarin 400 an axial resolution of about 100 nm is achieved. Resolutions even better than that could be achieved with two-photon excitable fluorophores operating at even deeper uv ranges. The axial secondary maxima are four times lower than in a single-photon 4Pi-confocal fluorescence arrangement. Furthermore, the two-photon type A 4Pi-confocal fluorescence microscope features all the advantages of two-photon microscopy such as operating at (biologically) less hazardous wavelengths and reduced amount of stray light. Photobleaching, and photodynamic damage are confined to the vicinity of the illumination spot. Following ref. [18], a laser power of the range of 1–50 mW should match the intensity requirements for two-photon excitation. The 4Pi-confocal fluorescent microscope has been set up and the predicted effects will be investigated employing a titanium-sapphire laser.

Further calculations show that for a type C two-photon 4Pi-confocal fluorescence microscope, the height of the axial side elevations are even below 5% of the main peak and a further resolution enhancement by 30 percent can be expected. Two-photon 4Pi-confocal fluorescence microscopy is a synergetic combination of two-photon excitation and 4Pi-confocal microscopy. It surpasses both the 4Pi-confocal fluorescence microscope by exploiting its full resolution enhancement potential and the two-photon microscope by featuring a resolution even higher than that of its single-photon excitation counterpart. Two-photon 4Pi-confocal fluorescence microscopy offers the best point resolution ever achieved in far-field microscopy.

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References